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Role of telomere length in subtelomeric gene expression and its possible relation to cellular senescence

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Transcriptional silencing of subtelomeric genes is associated with telomere length, which is correlated with age. Long and short telomeres in young and old people, respectively, coincide with gene repression and activation in each case. In addition, differential location of genes with respect to telomeres causes telomere position effect. There is very little evidence of the manner in which age-related telomere length affects the expression of specific human subtelomeric genes. We analyzed the relationship between telomere length and gene expression levels in fibroblasts derived from human donors at ages ranging from 0-70 years. We studied three groups of genes located between 100 and 150 kb, 200 and 250 kb, and >300 kb away from telomeres. We found that the chromatin modifier-encoding genes Eu-HMTase1, ZMYND11, and RASA3 were overexpressed in adults. Our results suggest that short telomere length-related overexpression of chromatin modifiers could underlie transcriptional changes contributing to cellular senescence. [BMB reports 2009; 42(11): 747-751]

INTRODUCTION

The best-known consequence of heterochromatin formation is repression of transcription, whether in the heterochromatin itself or in DNA regions adjacent to a heterochromatin domain. Telomeres are part of the heterochromatin, are composed of TTAGGG repeats (1), and possess a DNA double strand, as well as a 3' GT-rich single strand. In all linear chromosomes with telomeres, the single-strand overhang comprises an essential part of the chromosomal structure. Chromosome ends are protected from degradation by a T-loop (2), which keeps the DNA from breaking and preserves genome integrity (3). The formation of this T-loop depends on telomere-binding multiprotein complexes, which bind to telomeres and have fundamental role in gene regulation (4).

When telomeres become shorter, normal cells undergo an irreversible proliferation arrest. This phenomenon uncovered telomeres as regulators of a biological clock that activates senescence (5). It is unpractical to carry out *in vivo* studies on the mechanisms responsible for this biological process due to the long life of humans. An alternative is the use of cultured cells, given the generally accepted view that aging has a cellular origin.

Among diverse epigenetic phenomena, telomeres are implicated in transcriptional reversible gene silencing of nearby genes (6), leading to Position effect variegation (PEV) in *Drosophila* and Telomere position effect (TPE) in yeasts and humans (7). Subtelomeric genes suffer highly variable position effects depending on their proximity to telomeres and the protein complexes binding them (8). At present, it is accepted that telomere shortening affects expression in subtelomeric gene rich regions, leading to modifications in cell biology prior to the activation of TPE-mediated replicative senescence. In the same way as an epigenetic phenomenon such as DNA methylation, telomere shortening may be associated with some human diseases (9, 10). Although the loss of TPE is not a senescence trigger, it can be responsible for progressive changes in gene expression as a function of replicative age (11).

To date only one study reported TPE in telomeric human genes, whose expression could be influenced by alterations in the local heterochromatin structure (12). However, whether age-related telomere length correlates with the range of such chromatin alterations and how it affects the expression of specific human subtelomeric genes remain to be explored. The identification of genes influenced by telomere length would lead us to a better understanding of the mechanisms modulating cellular senescence. Therefore, in this work we addressed the expression of three sets of genes whose location varies with respect to telomeres, in fibroblasts derived from individuals from 0-70 years of age. We found that cells derived from adults present shorter telomeres, which correlates with cellular senescence as suggested by β-galactosidase expression. Analysis of genes located no more that 250 bp away from telomeres in adult cells revealed overexpression of ZMYND11 and Eu-HMTase1, which are implicated in restructuring and compacting chromatin, and of RASA3 which participates in proliferation and differentiation control. Our results suggest that the expression of chromatin modifiers, which are potentially involved in cellular proliferation and differentia-

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tion, is affected by telomere length, which in turn could constitute a means of regulating cellular senescence.

RESULTS

Fibroblast characteristics

Changes between both fibroblasts groups were evident from the time the culture started. The fibroblasts from adults over 60 years of age took longer to separate from tissue and adhere to a dish. Their growth rate to reach confluence was slower and presented characteristics of senescence. Although fibroblasts are generally spindle-shaped, many fibroblasts from adults became significantly larger in the cultures, changing in shape and physical appearance.

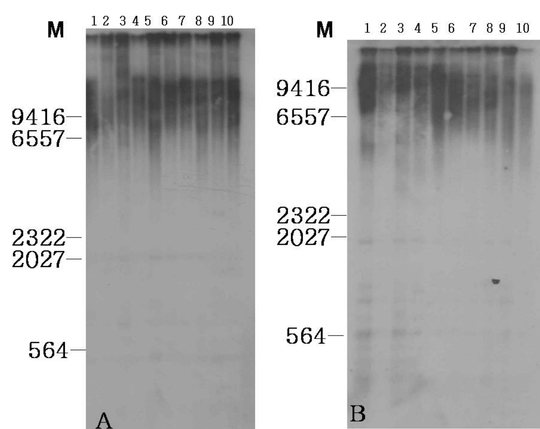

Fig. 1. Size of telomeres obtained by TRF. After digestion of the DNA from children (A) and adults (B), a smear was obtained that corresponds to the repeated telomeres (samples from each group were resolved in distinct gels). Adult telomeres were shorter than those of children (n = 20; $P \leq 0.05$; $\alpha = 0.001$, analyzed with Telometric 1.2 and SPSS 12.0 software programs). The telomeric probe was marked with ^{32}P. A λ HindIII weight marker was used.

Telomere length by terminal restriction fragment (TRF)

A decrease in telomere length was observed between groups (n = 20; $P \leq 0.05$; $\alpha = 0.001$). Average length in children and adults was 10,420.38 bp and 9,188.37 bp, respectively (Fig. 1).

Senescence-associated beta-galactosidase (SA-β-gal)

Senescent cells were present at all time points during the lifespan of the culture, but were a minor part when the culture was young. However, when the culture aged, a greater number of cells were positive to the β-gal stain (Fig. 2). Thus, as Rubin indicates (13), their serial propagation is accompanied by great changes in lisosomes, which grow progressively in number and show deep degenerative changes.

p16 expression levels

We found no differences in the expression levels of the p16 senescence marker between the group of children and adults. After culturing the sample of the youngest donor (26 days old) until it reached senescence (after a period of 11 months), a greater expression of p16 was observed in the final passage (subculture 21), as compared with one of the early passages (subculture 7), the latter being the time that population doublings diminished. There was no difference in the levels of expression of p16 when comparing cells from children and adults (0.033). However, an increase in its expression was found upon allowing the fibroblasts from the youngest donor to undergo senescence (3.398). Analysis was carried out with the Representational state transfer (REST) program.

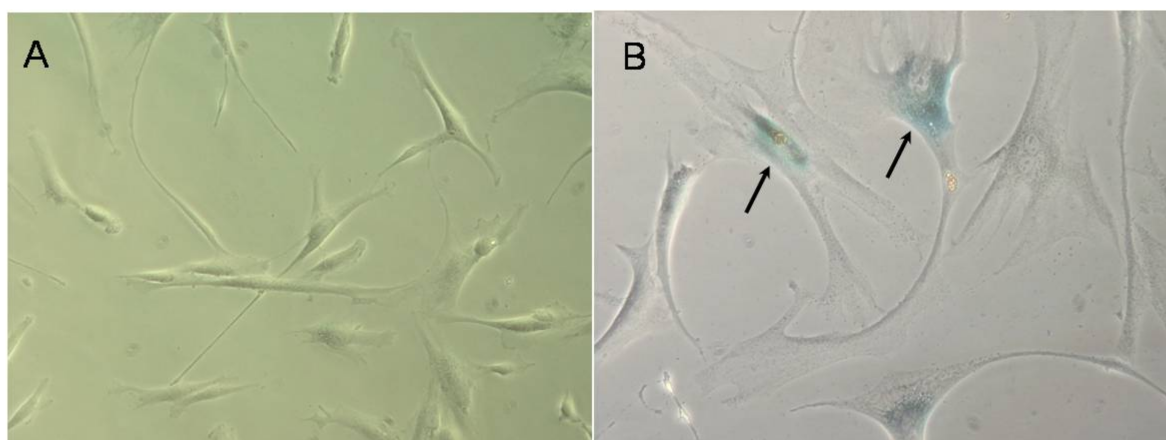

Fig. 2. Representative images of the fibroblasts studied. Cells from children (A) and adults (B). It can be observed that senescent fibroblasts from adults have a greater size and a flat appearance, while those from children are smaller and spindle-shaped. Fibroblasts from adults are stained blue by β-galactosidase (arrows), which indicates that they are senescent cells.

Table 1. Genes located in some subtelomeric regions

Regions	Gen	Abbreviation	Function	Expression significance (α)*
2q	Inhibitor of growth family, member 5	ING5	Tumor supressor	0.07
6q	PHD finger protein 10	PHF10	Unknown	0.24
9q	Euchromatic histone methyltransferase 1	EHMT1	Chromatin modification	0.037
10p	Adenovirus 5 E1A binding protein	BS69	Transcriptional repressor	0.009
12q	Zinc finger protein 10	ZNF10 (KOX1)	Transcriptional repressor	0.02
13q	RAS p21 protein activator 3	RASA3	Acting as a suppressor of RAS function	0.034
15q	BBP-like protein 2	BLP2	Regulatory roles in cell death or proliferation	0.33
16q	Growth arrest-specific 8	GAS8	Putative tumor supressor	0.091
20p	Defensin, beta 127	DEFB127	Immune response	0.22

*Significance was obtained with one way ANOVA test.

Real-time, quantitative Polymerase chain reaction (qRT-PCR)
We analyzed nine genes near the telomeres: Three genes between 100 and 150 kb (KOX1, DEFB127, BLP2); three genes between 200 and 250 kb (Eu-HMTase1, ZMYND11, RASA3), and three genes >300 kb from the telomeres (GAS8, PHF10, ING5). Four genes had a differential expression between child and adult groups (Table 1). Three of these are located between 200 and 250 kb from the telomere ($P \leq 0.05$). Only four overexpressed genes were found as follows: Eu-HMTase1 and ZMYND11, which regulate chromatin functions; RASA3, which regulates cellular proliferation and differentiation, and KOX1, which was only overexpressed in children and has been reported to participate in transcriptional repression.

DISCUSSION

Telomeres play a critical role in chromosomal structure and function, and are implicated in replicative senescence. It has been suggested that their shortening to a critical size acts as a mitotic clock. In addition to the difference in size observed between telomeres of children and adults, there were more senescent cells in cultures from adults and their number increased by age. It has been found that in many senescent cells, telomeres continue to be very long (5-10 kb), which suggests that entrance into senescence can be regulated more by 3' overhang size than by total length (14).

We found that SA-β-gal is related with the age of the donor; however, when we verified the senescence process with p16, we found no difference between the two groups. Palmero *et al.* (15) suggested that p16 overexpression could be related with progressive telomere shortening, but at the beginning of senescence.

In the case of the sample of the youngest donor, the telomeres did not present a critical length, which probably means that there was no increase in p16 transcription. When we cultured this sample until it reached senescence (21 passages and 11 months later), there was an increment in p16 expression level. This suggests that there were no differences in p16 expression in children and adults because adult telomeres continued to be sufficiently long for p16 activation to take place, which is in agreement with Smogorzewska and de Lange (16). Probably the presence of senescence cells is a natural phenomenon, although to date the number of senescence cells necessary to generate the aging of a tissue is unknown.

On the other hand, Wright and Shay (7) have proposed that changes in pre-senescent as well as senescent cells could be due to telomeric chromatin modification, and this may lead to silencing or desilencing of nearby genes. It has been suggested that the silencing gene transcriptional telomere link occurs because telomeric heterochromatin blocks a promoter, thus blocking access to transcription apparatus (17). In telomeres, as in other chromosomal regions, DNA repetitions cause heterochromatization, which in turn induces generalized transcriptional repression. This silencing (TPE) can be extended to large chromosomal regions (18) and is not specific for a particular gene. Of the nine genes analyzed in this study, four had differences in their expression levels, three presenting upexpression in adults and one in children. The transcript corresponding to the RASA3 gene codifies a member of the GAP1 family of activating proteins of GTPase and regulates the activity of RAS p21. All RAS genes act together as a suppressor of the RAS function, which controls cell proliferation and differentiation. The ZMYND11 gene, which codifies for a protein capable of binding to the adenovirus E1A protein, acts as a transcriptional repressor (19). Recently, it was suggested that this gene might participate in the restructuring of chromatin, as it also interacts with remodeling factors, including ATP-dependent helicases, histone desacetylases, and histone methyltransferases (20). In addition to this function, nothing else is known about this gene. Finally Eu-HMTase1, also found overexpressed, might regulate mono- and dimethylation of H3-K9 in the euchromatin. This gene, along with G9a (another methyltransferase), directs the recruitment of HP1 proteins to the euchromatin (21). It is found overexpressed in adults and can induce the repression of diverse genes. It is interesting that of the overexpressed genes in adults, two are implicated in chromatin restructuring. The number of chromosomes that are required to

have short telomeres for senescence to begin is not yet known, nor is the extent to which telomeric structures lead to silencing. Further studies are necessary in order to conduct an analysis of changes in the heterochromatin.

MATERIALS AND METHODS

Cell lines and culture procedures

Fibroblast cell lines were established from abdominal skin biopsies from 20 healthy individuals. Ten cell lines were derived from children between 26 days and 12 years of age, and 10 lines from adults between 40-70 years of age. All fibroblast cell lines were cultured in DMEM supplemented with 10% bovine fetal serum (Gibco-Invitrogen), 100 μg/ml penicillin, and 100 U/ml streptomycin (Invitro). All tests were performed with prior informed consent.

DNA and RNA isolation

We obtained DNA and RNA from cells cultured for five passages. DNA was obtained using the method described by Gross-Bellard *et al*. (22), and total RNA was obtained with TRIZOL® (Invitrogen).

Terminal restriction fragment length measurement (TRF)

We employed the method described by Baur *et al*. (23). In brief, DNAs were digested with 20 U of HinfI, HaeIII, and RsaI (New England BioLabs). Restriction products were electrophorezed in 0.8% agarose gels during 20 h at 40 V to obtain appropriate telomere-fragment separation. DNA was transferred to Hybond N+ membrane (Amersham Life Science). The membrane was blotted with a telomeric ^{32}P probe. Average telomeric length was determined by densitometric analysis of autoradiographies using Telometric 1.2 software (*BioTechniques* Software Library) and the SPSS 12.0 software program (Chicago, IL, USA).

Senescence associated-β galactosidase activity (SA-β gal)

The β-gal activity was determined by the method described by Dimri *et al*. (24). Once cells reached 5 passages, they were washed with PBS, fixed in 2% formaldehyde/0.2% glutaraldehyde PBS, for 5 min at room temperature, washed in PBS and stained overnight at 37°C. The β-gal positive cells showed blue precipitate in the cytoplasm.

p16 expression

The expression of this gene was determined by qRT-PCR. The primers used were described by Satyanarayana *et al*. (25). The amplification was carried out as for the genes near telomeric ends.

qRT-PCR

cDNA was synthesized from 1 μg of total RNA from each donor using the Thermoscript™ RT-PCR System (Invitrogen, Carlsbad, CA, USA). Real time gene expression analyses were done with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA), using 250 ng of cDNA, in a Rotor-Gene 6 (Corbett Research) thermocycler. PCR conditions were as follows: 95°C for 2 min followed by 37 cycles of 95°C for 15 sec, 50-53°C for 15 sec, acquisition at 72°C for 15 sec, and the product-melting cycle. All primers were designed with OLIGO 4.1 (Table 2). Duplicate reactions were prepared for each sample, together with negative (H_2O control) and positive control (HPRT gene). A standard curve was used with these assays, and Rotor-Gene 6 data analysis software (Corbett Research) was utilized in all processes.

Statistical analysis

Telomere length changes between adults and children cells

Table 2. Designed primers with the sofware OLIGO™ 4.0

Chromosome	Gene	Accession	Primers (20 bp)	Tm °C	Length (bp)
2q	ING5	NM_032329	5′-gaa gat aag aaa gca gag at-3′ 5′-act ttg tcg tca ctg tat tc-3′	50.4	143
6q	PHF10	NM_018288	5′-cga aag atg ata atg aag at-3′ 5′-atc aat cac ttc atc act gc-3′	50.2	362
9q	EHMT1	NM_024757	5′-aga tcc cag aga agt tcg ag-3′ 5′-gtt ccc att ctt cgt ttt tt-3′	52.1	228
10p	BS69	NM_006624	5′-tgt gac ctg tgt ttt cgt gt-3′ 5′-ccc atc tcc tgt ttg ttt gt-3′	50.8	134
20p	DEFB127	NM_139074	5′-acc cac agt aac cga aca ac-3′ 5′-tga gaa gca agt gaa aac ga-3′	50.9	292
12q	ZNF10 (KOX1)	NM_015394	5′-cct ggt ttc ctt ggg tta tc-3′ 5′-tca ttc ctt gcc att cct tc-3′	52.5	213
13q	RASA3	NM_007368	5′-cca aaa acc ttc cct ctt ac-3′ 5′-gta gaa gga cag gtg acg aa-3′	52.6	176
16q	GAS8	NM_001481	5′-agt gag gtg gtg gtg aag aa-3′ 5′-cat tct tcc tct cct cca ct-3′	52.8	184
15q	BLP2	NM_025141	5′-ata agt ggt cta cgg ctc tg-3′ 5′-cac acc aca tca act cct ac-3′	53.8	266

were compared by one-Way ANOVA. A P value of ≤ 0.05 was considered significant. A statistical software package (SPSS 12.0, SPSS, Chicago, IL, USA) was used for analysis.

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